Supplementary Figures

Immunotherapeutic IL-6R and Targeting the MCT-1/IL-6/CXCL7/PD-L1 Circuit Prevent Relapse and Metastasis of Triple-Negative Breast Cancer

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Supplementary Fig. S1: Source Data Fig. 1K

GSEA MSigDB Hallmark gene set: shMCT-1 vs. scramble (NC)

NAME	SIZE	NES	NOM *p*-val	FDR *q*-val
INTERFERON_ALPHA_RESPONSE	89	-2.82	0.000	0
INTERFERON_GAMMA_RESPONSE	185	-2.66	0.000	0
IL6_JAK_STAT3_SIGNALING	83	-1.79	0.000	0.005
ESTROGEN_RESPONSE_LATE	193	-1.73	0.000	0.007
HEME_METABOLISM	185	-1.67	0.000	0.009
P53_PATHWAY	192	-1.66	0.000	0.009
COMPLEMENT	184	-1.65	0.000	0.008
APOPTOSIS	156	-1.63	0.000	0.01
INFLAMMATORY_RESPONSE	196	-1.62	0.000	0.009
APICAL_SURFACE	42	-1.6	0.011	0.012
ALLOGRAFT_REJECTION	186	-1.6	0.000	0.011
HYPOXIA	193	-1.56	0.001	0.014
KRAS_SIGNALING_UP	194	-1.55	0.000	0.014
PROTEIN_SECRETION	94	-1.54	0.004	0.015
COAGULATION	132	-1.41	0.026	0.056
XENOBIOTIC_METABOLISM	189	-1.41	0.008	0.056
TNFA_SIGNALING_VIA_NFKB	193	-1.37	0.016	0.071
ESTROGEN_RESPONSE_EARLY	195	-1.35	0.021	0.085
ADIPOGENESIS	192	-1.34	0.023	0.084
APICAL_JUNCTION	193	-1.32	0.036	0.094
GLYCOLYSIS	196	1.29	0.029	0.092
DNA_REPAIR	147	1.46	0.004	0.027
MTORC1_SIGNALING	194	1.78	0.000	0.002
G2M_CHECKPOINT	187	2.02	0.000	0
CHOLESTEROL_HOMEOSTASIS	71	2.23	0.000	0
E2F_TARGETS	195	2.36	0.000	0
MYC_TARGETS_V1	193	2.45	0.000	0
MYC_TARGETS_V2	58	2.49	0.000	0

Supplementary Fig. S1. GSEA of the hallmark molecular signatures database (MSigDB) as appeared in bubble plots (Fig. 1K). The statistical threshold was set at $p < 0.05$ (normal (NOM) *p*-val) and false discovery rate (FDR) $q < 25\%$ (FDR *q*-val). NES: Normalized enrichment score.

Supplementary Fig. S2

Important <u>downregulated genes</u> in metastasis and IL-6/EGFR/PD-L1 signaling

(as appears in volcano plots (Fig. 1L)

Gene Ontology: Biological Process	Gene Symbol	Functions (Description from GeneCards)	p-value	Fold-Change
morphogenesis of an epithelium	*Car9*	Reversible hydration of carbon dioxide. Participates in pH regulation. May be involved in the control of cell proliferation and transformation.	0.0325	-1.53
angiogenesis	*Ang2*	Facilitate endothelial cell migration and proliferation, thus serving as a permissive angiogenic signal.	0.0479	-1.47
microtubule-based process	*Tubb2a*	Major constituent of microtubules, key participants in processes such as mitosis and intracellular transport	0.0280	-1.43
N-acetylneuraminate metabolic process	*St6gal1*	Transfers sialic acid from CMP-sialic acid to galactose-containing acceptor substrates. Involved in the generation of the cell-surface carbohydrate determinants	0.0307	-1.43
negative regulation of endopeptidase activity	*Serpina3i*	Inhibit neutrophil cathepsin G and mast cell chymase, both of which can convert angiotensin-1 to the active angiotensin-2.	0.0416	-1.35
MAPK cascade	*Epgn*	Promotes the growth of epithelial cells. May stimulate the phosphorylation of EGFR and mitogen-activated protein kinases.	0.0450	-1.35
epidermal growth factor receptor signaling pathway	*Areg*	Ligand of the EGF receptor/EGFR. Autocrine growth factor as well as a mitogen for a broad range of target cells including astrocytes, Schwann cells and fibroblasts.	0.0055	-1.32
adaptive immune response	*Ifna4*	Have antiviral activities. Stimulates the production of two enzymes: a protein kinase and an oligoadenylate synthetase.	0.0447	-1.26
protein glycosylation	*B3gnt4*	Beta-1,3-N-acetylglucosaminyltransferase involved in the synthesis of poly-N-acetyllactosamine. Has activity for type 2 oligosaccharides.	0.0159	-1.25
retina homeostasis	*Cdh3*	Calcium-dependent cell-cell adhesion protein, comprised of five extracellular cadherin repeats. Involved in loss of heterozygosity events in breast and prostate cancer.	0.0024	-1.20

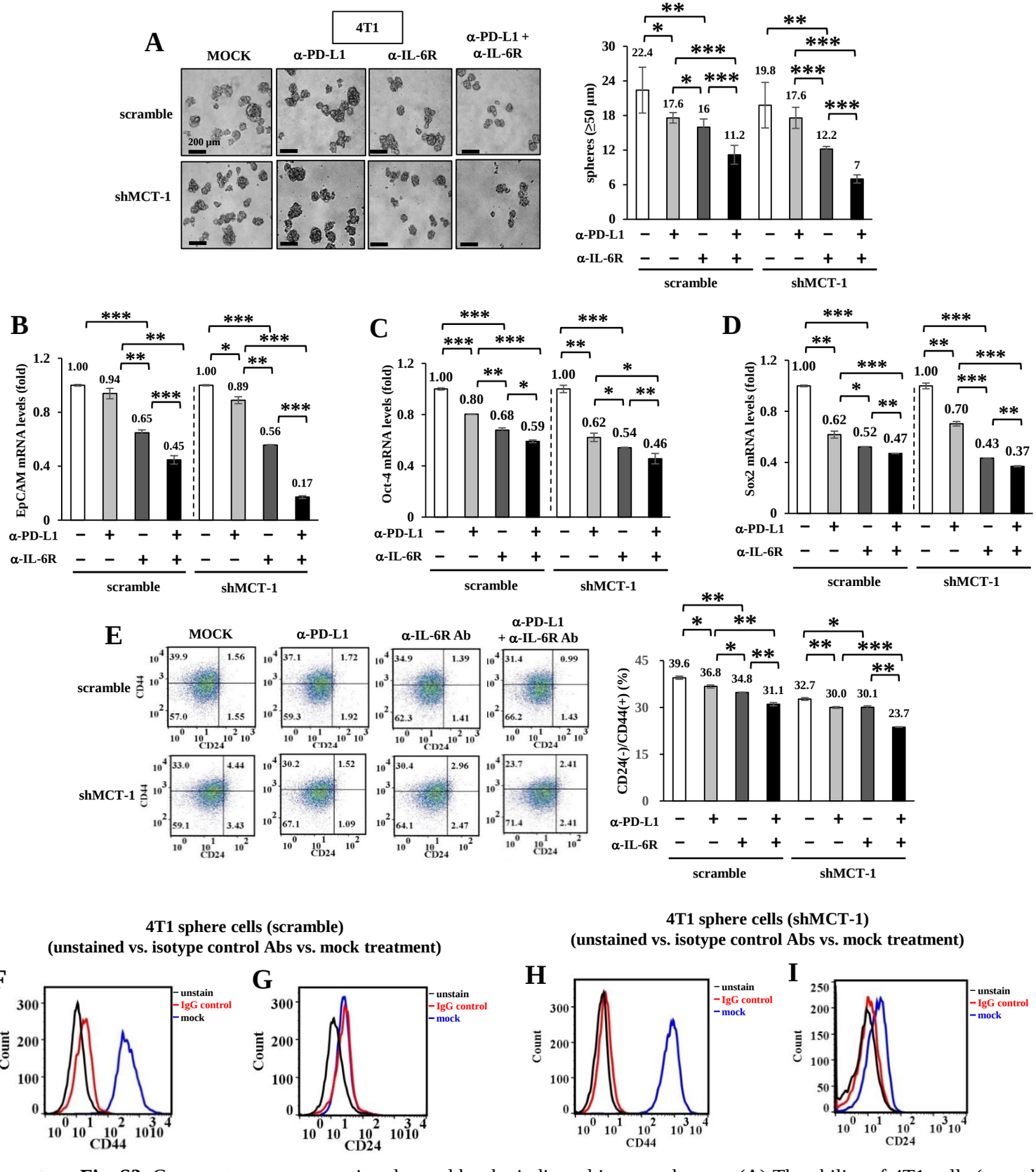
Important <u>upregulated genes</u> in metastasis and IL-6/EGFR/PD-L1 signaling

(as appears in volcano plots (Fig. 1L)

Gene Ontology: Biological Process	Gene Symbol	Functions (Description)	p-value	Fold-Change
positive regulation of defense response to virus by host	*Cldn7*	Plays a major role in tight junction-specific obliteration of the intercellular space.	0.0128	1.97
response to molecule of bacterial origin	*Cxcl2*	Involved in immunoregulatory and inflammatory processes. Suppresses hematopoietic progenitor cell proliferation in vitro.	0.0218	1.71
ND	*Mall*	Machinery for raft-mediated trafficking in endothelial cells.	0.0042	1.58
MyD88-independent toll-like receptor signaling pathway	*Tnip3*	Binds to zinc finger protein TNFAIP3 and inhibits NF-kappa-B activation induced by tumor necrosis factor, Toll-like receptor 4 (TLR4), interleukin-1 and 12-O-tetradecanoylphorbol-13-acetate.	0.0156	1.46
cell adhesion	*Ly9*	Self-ligand receptor of the signaling lymphocytic activation molecule (SLAM) family. Involved in the regulation and interconnection of both innate and adaptive immune response.	0.0200	1.27

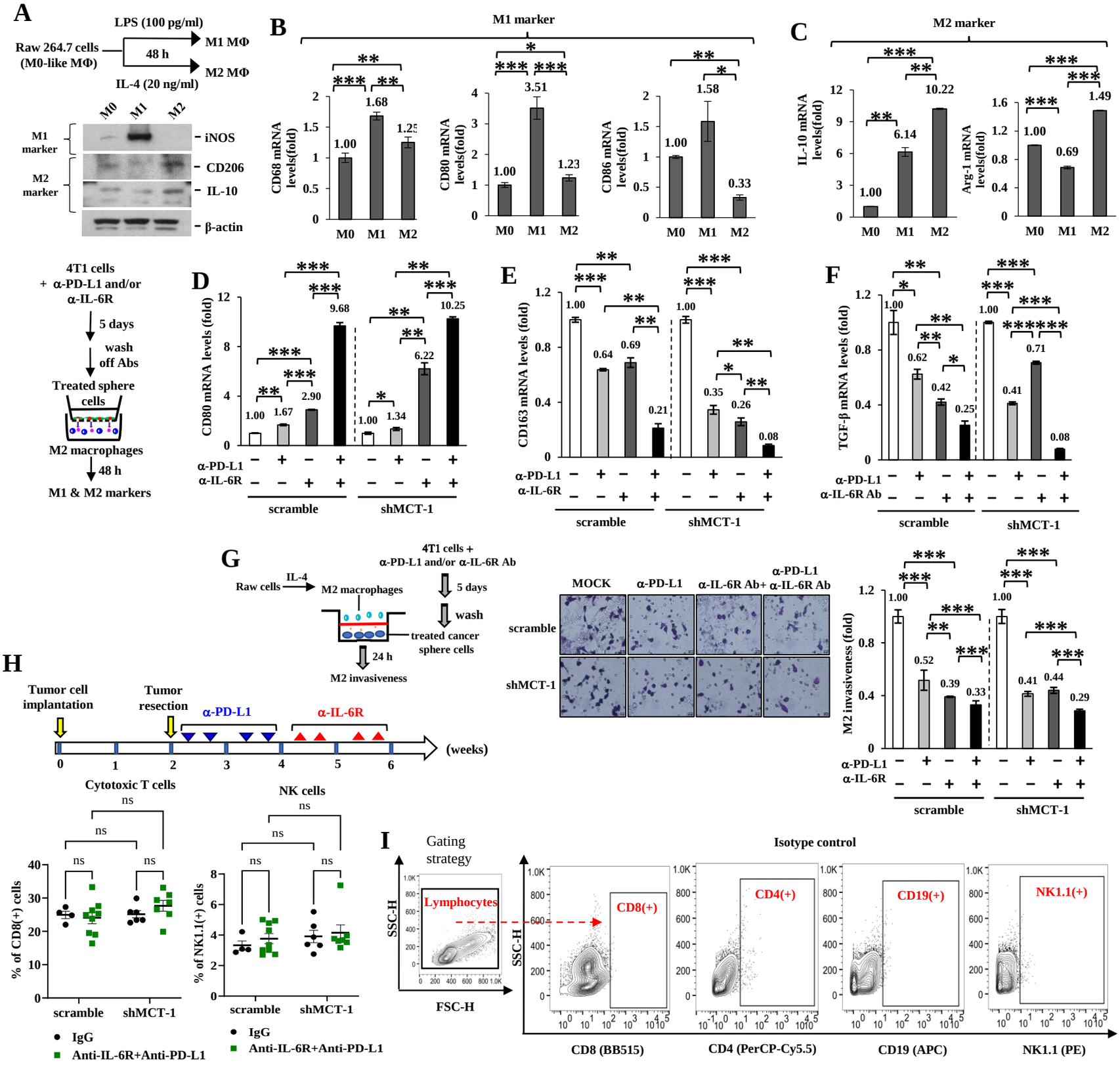
Supplementary Fig. S2. Deregulated genes related to metastasis and IL-6/EGFR/PD-L1 signaling. The shMCT-1 cells were compared with scramble cells (p<0.05 and fold change ≥ 1.2 or <1.2) as appeared in volcano plots (Fig. 1L).

Supplementary Fig. S3



Supplementary Fig. S3. Cancer stemness properties changed by the indicated immunotherapy. (A) The ability of 4T1 cells (scramble vs. shMCT-1) to form mammospheres was measured following anti-mouse IL-6R Ab and/or anti-human PD-L1 Ab (atezolizumab) treatment for 5 days (n=5). Scale bar, 200 μm. (B-D) EpCAM (B), Oct4 (C) and Sox2 (D) expression levels were analyzed by qRT-PCR (n=5). (E) CD24(-)/CD44(+) subpopulations in the immunotherapy-treated spheres were analyzed by flow cytometry (n=5). (F-I) Flow cytometry analysis of unstained, IgG2b, κ-treated and MOCK-treated 4T1 cells of CD44(+) (F, H) or CD24(+) (G, I) populations in the scramble (F-G) and the shMCT-1 (H-I) conditions. Data are presented as the mean ± SEM. One-way ANOVA with the Tukey-Kramer post hoc test was used to evaluate the statistical significance of pairwise comparisons.

Supplementary Fig. S4



Supplementary Fig. S4. Immunotherapy of BCSCs inhibits M2 macrophage polarity and invasiveness. Murine Raw264.7 M0-like macrophages were treated with LPS (100 pg/ml) or IL-4 (20 ng/ml) for 48 h to induce M1 and M2 polarization, respectively. (A) Intracellular markers for M1 (iNOS) and M2 (CD206 and IL-10) macrophages were examined in Raw264.7 M0-like macrophages and the polarized M1 and M2 macrophages. (B-C) The mRNA expression levels of M1 (CD68, CD80 and CD86) (B) and M2 (IL-10 and Arg-1) (C) markers were determined by qRT-PCR (n=5). (D-F) Anti-IL-6R and/or anti-PD-L1 immunotherapy-pretreated BCSCs were placed in the upper chamber and used to define the macrophage polarity as cocultured with the Raw 264.7 M2 macrophages in the lower chamber for 48 h. M1 (CD80) (D) and M2 (CD163 and TGF-β) (E-F) markers were determined by qRT-PCR (n=5). (G) The M2 macrophages penetrating through the upper chamber of an invasion transwell were evaluated (n=5) after coculture with the immunotherapy-treated BCSCs in the lower chamber for 24 h. (H) Splenic CD8(+) CTLs and NK cells were detected in the 4T1 xenograft mice after the sequential immunotherapy (anti-PD-L1 → anti-IL-6R). (I) Flow cytometry was examined immune cells detected by isotype control Abs (n=5). Data are presented as the means ± SEM. One-way (B-G) and two-way (H) ANOVA with the Tukey-Kramer post hoc test were used to calculate statistical significance.

Supplementary Fig. S5. Primer sequences for specific amplicons.

Gene		Human (*Homo sapiens*)
β-actin	F	5′-CACCAGGGCGTGATGGTGGG-3′
	R	5′-GATGCCTCTCTTGCTCTGG GC-3′
CXCL7	F	5′-CTGACTGCTCTGGCTTCCTC-3′
	R	5′-ATGCAGCGGAGTTCAGCATA-3′
IL-6	F	5’- ATTCCTTCTTCTGGTCAGAAACC-3’
	R	5’-ACAAAGGATATTCAAACTGCATAGC-3’
MCT-1	F	5’-AGGCATTATCTTCATGCTGTCA-3’;
	R	5’-AATGATGGGCTGTGGCATAT-3’
PD-L1	F	5’-GTTGAAGGACCAGCTCTCCC-3’
	R	5’-CTTGTAGTCGGCACCACCAT-3’

Gene		Mouse (*Mus musculus*)
EpCAM	F	5’-TGCTGGAATTGTTGTGCTGG-3’
	R	5’-AGA TGTCTTCGTCCCACG-3’
Oct4	F	5’-TGA CCGCATCTCCCCTCTAA-3’
	R	5’-TCTTCCCAGAGGGAGCTCAA-3’
SOX2	F	5’-CATGAAGGAGCACCCGGATT-3’
	R	5’-TTAATGTGCGCGTAACTGTG-3’
Areg	F	5’-AGAACTCCGCTGCTACCGCT-3’
	R	5’-AGCTCAAGTCCACCGGCACT-3’
Epgn	F	5’-TGCGATTGGGATTGGCGTCG-3’
	R	5’-ATGGGCTCCCTCCAGAGCAG-3’
B3gnt4	F	5’-CCTTCGGAGTGTGCCAGGGA-3’
	R	5’-CACCAGCTTCAGCTGCCGAC-3’
St6gal1	F	5’-ATGGGTGCCTACCATCCGCT-3’
	R	5’-TCCGGAAGCCAGACAGGGTA-3’
Serpina3i	F	5’-ATGCCCTTTGACCCCCGTGA-3’
	R	5’-GCAGGACAGCTCATCATCCCG-3’
Ang2	F	5’-ATGGCGATGAGCCCAGGTCC-3’
	R	5’-CTGTCGTCCCGGCCTTTTGG-3’

Gene		Mouse (*Mus musculus*)
β-actin	F	5’-ATGGTCTTGGTTTCTGTGCCT-3’
	R	5’-CCACATCAAAGGCCGTGTTC-3’
PPIA	F	5’-CATCACGGCCGATGACGAGC-3’
	R	5’-TCCAGTGCTCAGAGCTCGAAAGT-3’
CD68	F	5’-ACACTTCGGGCCATGTTTCT-3’
	R	5’-GGGGCTGGTAGGTTGATTGT-3’
CD80	F	5’-ATGCTCACGTGTCAGAGGAC-3’
	R	5’-TGACAACGATGACGACGACT-3’
CD86	F	5’-GCAAGGTCACCCGAAACCTA-3’
	R	5’-CACACACCATCCGGGAATGA-3’
Adgre1	F	5’-TAGCGGGGATGTTGCACTAC-3’
	R	5’-TTGTGGCCAGCTATTGCAGA-3’
Arginase 1 (Arg1)	F	5’-GGTCTGTGGGGAAAGCCAAT-3’
	R	5’-CAGTGTGAGCATCCACCCAA-3’
IL-10	F	5’-CAGTACAGCCGGGAAGACAA-3’
	R	5’-AGGCTTGGCAACCCAAGTAA-3’
TGF-β	F	5’-TGTACGGCAGTGGCTGAACC-3’
	R	5’-CATGGATGGTGCCCAGGTCG-3’
CD163	F	5′-CCG GGAGATGAATTCTTGCCT-3′
	R	5′-AGACACAGAAATTAGTTCAGCAGCA-3′
Foxp3	F	5’-CCTGGTTGTGAGAAGGTCTTCG-3’
	R	5’-TGCTCCAGAGACTGCACCACTT-3’

Table S1

Table S1. Microarray analysis of DEGs. Each worksheet tab represents selected de-enriched hallmark gene sets in shMCT-1 cells identified by 1,000 permutations by GSEA: (1) INTERFERON_ALPHA_RESPONSE; (2) INTERFERON_GAMMA_RESPONSE; (3) IL6_JAK_STAT3_SIGNALING; and (3) INFLAMMATORY_RESPONSE. Genes in the data set are ordered by their position in the ranked list of genes. Genes with a 'Yes' value in the core enrichment column contribute most to the enrichment result. (XLS 51 KB)

Table S2

Table S2. RNA-seq experiments identify DEGs. Each worksheet tab represents pairwise comparison between the groups: (1) shMCT-1 cells treated with anti-IL-6R vs. scramble cells treated with anti-IL-6R (sht_vs_sct); (2) shMCT-1 cells treated with anti-IL-6R vs. scramble cells treated with IgG (sht_vs_sc); (3) shMCT-1 cells treated with anti-IL-6R vs. shMCT-1 cells treated with IgG (sht_vs_sh); (4) scramble cells treated with anti-IL-6R vs. scramble cells treated with IgG (sct_vs_sc); and (5) shMCT-1 cells treated with IgG vs. scramble cells treated with IgG (sh_vs_sc), respectively. Genes were assigned using Ensembl ID and gene symbol. Normalized gene abundance was listed as trimmed mean of M-values (TMM). Fold change ($\log_2$) and *p*-value from the Fisher's exact test statistical analysis are shown. (XLS 2.2 MB)